IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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In re Application of:	Group Art Unit: 1651
Kazuhisa MUKAI et al.	Examiner: Lora E. Barnhart
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For: PROCESS FOR PRODUCING...	November 28, 2008

DECLARRATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

1. I, Tomoyuki NISHIMOTO, declare as follows:

2. I am a citizen of Japan, residing at 500-30 Meguro-cho, Okayama-shi, Okayama, Japan.

3. In 1985, I received a bachelor of Agriculture from Osaka Prefecture University, and in 1998 I received a doctorate of Agriculture from the above-identified university.

4. As shown in my curriculum vitae attached hereto as Attachment A, from 1990 to 2004, I researched in Hayashibara Biochemical Laboratories Inc. fundamental studies and industrial applications of carbohydrates and their related enzymes. Since 2004, I have been a chief scientist of Research Center, Hayashibara Biochemical Laboratories, Inc.

5. I have read and thoroughly understood the present invention and the content of the United States Patent No. 5,137,723, titled "α-GLYCOSYL-L-ASCORBIC ACID, AND ITS PREPARATION AND USES" applied for by "Kabushiki Kaisha Hayashibara Seibutsu Kagaku Kenkyujo" (Hayashibara Biochemical Laboratories, Inc.), cited in an official action in the procedure of the present invention.

6. To demonstrate a significant difference in the productivity of 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA2G), 5-O-α-D- glucopyranosyl-L-ascorbic acid (AA5G), and 6-O-α-D-glucopyranosyl-L-ascorbic acid (AA6G) between the α-isomaltosyl glucosaccharide-forming enzyme (abbreviated as "IMG", hereinafter) according to the present invention and rat intestine α-glucosidase (RIAGase) described in the above-identified patent, I conducted the following experiments.

7. IMG according to the present invention

Experiment 7-1: Cultivation of *Arthrobacter globiformis* A19 (FERM BP-7590)

According to the method described in Experiment 1 of the specification of the present invention, *Arthrobacter globiformis* A19 (FERM BP-7590) was cultured in 4 liters of a liquid culture medium in a 5 liter-fermenter at 27°C for 48 hours under aeration-agitation conditions. After centrifuging the culture broth, the resulting 3.7 liters supernatant had about 0.98 unit/ml of IMG activity and used as a crude IMG.

The activity of IMG was measured by the same method as described in Experiment 1 of the specification of the present invention.

Experiment 7-2: Partial purification of IMG

IMG in the supernatant was partially purified by successive salting out and column chromatography using "DEAE-TOYOPERAL® 650S" gel, commercialized by Tosoh Corporation, Tokyo, Japan, according to Experiment

2 of the specification of the present invention. As a result, a partially purified IMG specimen with a specific activity of 30.6 units/mg-protein was obtained.

8. Rat intestine α-glucosidase (RIAGase)

Experiment 8-1: Preparation of crude RIAGase

Crude RIAGase was prepared using "INTESTINAL ACETONE POWDERS FROM RAT", Lot. No. 109F8105, commercialized by Sigma-Aldrich Japan K.K., as a material according to the method described in EXPERIMENT 1 of US Patent No. 5,137,723 with a slight modification. One gram of the acetone powder was added to 20 ml of 50 mM phosphate buffer (pH 7.0), fed to homogenizer, and centrifuged, after which the supernatant was admixed with 4 mg trypsin, and allowed to stand at ambient temperature for 2 hours. The resulting solution was admixed with 2 volumes of a chilled ethanol. The formed sediment was collected by centrifugation, dissolved in 10 mM acetate buffer (pH 6.0), and dialyzed against the same acetate buffer as above. The dialyzed solution (11.8 ml) was used as a crude RIAGase.

Experiment 8-2: Partial purification of RIAGase

The crude RIAGase obtained in Experiment 8-1 was chromatographed on a column of "DEAE-TOYOPEARL® 650S" gel, commercialized by Tosoh Corporation, Tokyo, Japan, in usual manner to collect RIAGase-active fraction. As a result, a partially purified RIAGase specimen with a specific activity of 34.2 units/mg-protein was obtained.

The activity of RIAGase was measured by the same method as described in EXPERIMENT 1 of US Patent 5,137,723.

9. Formation of AA2G by the enzymes

Experiment 9-1: Enzymatic reaction

An aqueous solution containing 5% (w/v) of L-ascorbic acid and 5% (w/v) of "PINEDEX® #1", a partial starch hydrolyzate commercialized

by Matsutani Chemical Industries Co., Ltd., Hyogo, Japan, as a glucosyl donor, adjusted to pH 5.3 (Substrate solution A) was used as a substrate for IMG and RIAGase. To Substrate solution A, 5, 10, or 20 units/g-glucosyl donor of the partially purified IMG specimen prepared in Experiment 7-2; or 2.5, 5, 10, or 20 units/g-glucosyl donor of the partially purified RIAGase specimen prepared in Experiment 8-2; was added and subjected to an enzyme reaction at 50°C for 24 hours. After the reaction, the reaction mixture was boiled for 10 min to inactivate IMG or RIAGase.

In addition, in the case of RIAGase, an aqueous solution containing 5% (w/v) of L-ascorbic acid and 5% (w/v) of "MALTOSE HHH", a reagent grade hydrous crystalline maltose with a purity of 99.9% (w/w) or higher, commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan, as a glucosyl donor, adjusted to pH 5.3 (Substrate solution B) was also used as a substrate and the enzyme reaction was carried out by the same procedure as above.

Then, each reaction mixture, obtained by allowing IMG or RIAGase to act on the substrate, was admixed with 40 units/g-partial starch hydrolyzate or maltose of glucoamylase commercialized by Seikagaku Corporation, Tokyo, Japan, to hydrolyze the remaining partial starch hydrolyzate or maltose into glucose, and subjected to an enzyme reaction at 40°C for 17 hours. After the reactions, each reaction mixture was boiled for 10 min to inactivate glucoamylase. The resulting each solution was subjected to high performance liquid chromatography (HPLC).

Experiment 9-2: Contents of AA2G, AA5G, and AA6G in the reaction mixture

According to the method described in Experiments 5 and 7 of the specification of the present invention, contents of AA2G, AA5G, and AA6G in the reaction mixtures were determined by HPLC using the following conditions:

(HPLC conditions)

Column: "WAKOPAK WB-T-330", a column commercialized by Wako Pure

Chemical Industries, Ltd., Osaka, Japan;

Column temperature: 25°C;

Solvent: 70 ppm of aqueous nitrate solution;

Flow rate: 0.5 ml/min;

The contents of L-ascorbic acid, AA2G, AA5G, and AA6G, on a dry solid basis were determined by measuring those absorbance at 238 nm using "UV-8020", a spectrophotometer commercialized by Tosoh Corporation, Tokyo, Japan; and measuring the composition of the reaction mixture including those using "RI-8020", a refractive index detector commercialized by Tosoh Corporation, Tokyo, Japan.

10. Experimental results

The results of Experiment 9 are summarized in Table 1 and Fig. 1.

Table 1

Enzyme	Glucosyl donor	Amount of Enzyme (units/g-donor)	Content in the reaction mixture (%, on a dry solid basis)		
			AA2G	AA5G	AA6G
IMG*	PINEDEX #1**	5	22.1	ND***	ND***
		10	24.5	ND***	ND***
		20	25.3	ND***	ND***
RIAGase	PINEDEX #1**	2.5	9.8	0.6	ND***
		5	13.0	1.1	0.2
		10	14.9	2.0	0.3
		20	13.0	3.4	0.7
	Maltose HHH	2.5	9.1	0.6	ND***
		5	13.7	1.1	0.2
		10	18.6	1.9	0.3
		20	14.7	3.1	0.6

*: IMG from *Arthrobacter globiformis* A19 (FERM BP-7590)

**: A partial starch hydrolyzate commercialized by Matsutani Chemical Industries Co., Ltd., Hyogo, Japan

***: Not detected

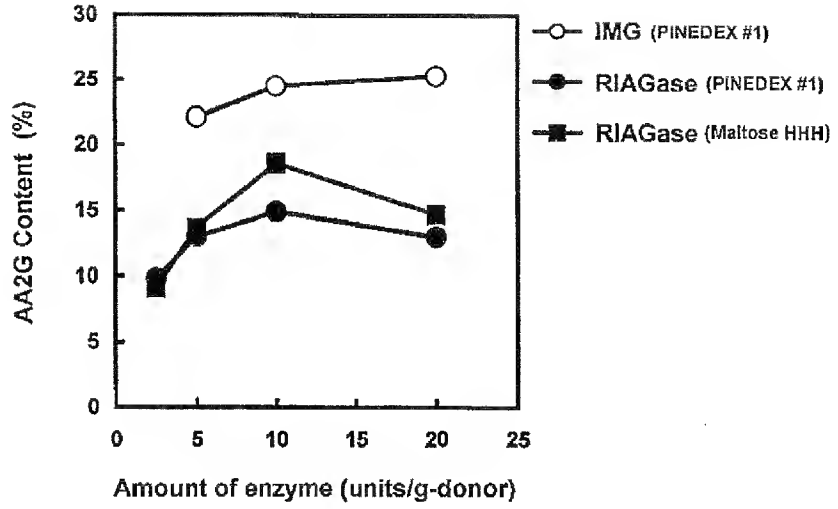

Fig. 1

As evident from the results in Table 1 and Fig. 1, AA2G content in the reaction mixture reached about 25%, on a dry solid basis, when IMG from *Arthrobacter globiformis* A19 (FERM BP-7590) was allowed to act on the partial starch hydrolyzate. In addition, AA5G and AA6G, as by-products, were not detected in the reaction mixture.

On the contrary to this, when RIAGase was allowed to act on the partial starch hydrolyzate, the maximum AA2G content in the reaction mixture was only about 15%, on a dry solid basis. Further, RIAGase produced AA5G and AA6G, as by-products, in amounts of 2.0% and 0.3%, respectively. In addition, when RIAGase was allowed to act on maltose which is the best substrate for RIAGase, the maximum AA2G content in the reaction mixture was only about 19%, on a dry solid basis, and the contents of AA5G and AA6G, as by-products, were 1.9% and 0.3%, respectively.

11. Conclusion:

The above experimental results indicate that the IMG from *Arthrobacter globiformis* A19 (FERM BP-7590) is a significantly different

enzyme from RIAGase in the productivity of AA2G. Further, the IMG is superior to RIAGase for the efficient production of AA2G because it does not produce AA5G and AA6G, as by-products, in the reaction mixture.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Tomoyuki Nishimoto

NAME: Tomoyuki NISHIMOTO

28th day of November, 2008

DATE: 28th day of November, 2008

Attachment A

CURRICULUM VITAE

Name:	Tomoyuki NISHIMOTO
Affiliation:	Hayashibara Biochemical Laboratories, Inc., 675-1, Fujisaki, Okayama-shi, Okayama, Japan 702-8006 Tel: +81 86 276 8670
Date of Birth:	December 26, 1961
Education:	Granted and received a bachelor from Osaka Prefecture University, Agricultural Department in 1985. Granted and received a master's degree from Osaka Prefecture University, Agricultural Department in 1987. Received a doctorate of Agriculture at Osaka Prefecture University in 1998.

Brief Chronology of Employment:

1987 (April)	Researcher, Hayashibara Co., Ltd.
1987 (July)	Researcher, Toyama Medical and Pharmaceutical University, under the employment of Hayashibara Biochemical Laboratories, Inc.
1990	Researcher, Amase Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.
2002	Senior Scientist, Amase Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.
2004	Chief Scientist, Amase Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.
2006	Chief Scientist, Glycoscience Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.

Public Employment:

2006 (September)-	Member of Editorial Board of Journal Applied Glycoscience

Attachment B

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